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Cellular responses to recurrent pentylenetetrazole-induced seizures in the adult zebrafish brain

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Abstract

A seizure is a sustained increase in brain electrical activity that can result in loss of consciousness and injury. Understanding how the brain responds to seizures is important for development of new treatment strategies for epilepsy, a neurological condition characterized by recurrent and unprovoked seizures. Pharmacological induction of seizures in rodent models results in a myriad of cellular alterations, including inflammation, angiogenesis, and adult neurogenesis. The purpose of this study is to investigate the cellular responses to recurrent pentylenetetrazole seizures in the adult zebrafish brain. We subjected zebrafish to five once daily pentylenetetrazole induced seizures and characterized the cellular consequences of these seizures. In response to recurrent seizures, we found histologic evidence of vasodilatation, perivascular leukocyte egress and leukocyte proliferation suggesting seizure-induced acute CNS inflammation. We also found evidence of increased proliferation, neurogenesis, and reactive gliosis. Collectively, our results suggest that the

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Competing Interests

The authors have no competing interests to disclose.

Contributions

Phan Q. Duy designed and directly performed all experiments used in data collection for this manuscript. Phan Q. Duy also performed all pertinent data analysis and extensively wrote major parts of the manuscript, including the “Methods”, “Results”, and “Discussion” section.

Michael Berberoglu PhD directly supervised the performance of all experiments used in data collection for this manuscript and assisted in data analysis and manuscript preparation.

Christine Beattie PhD supervised the performance of these experiments and greatly assisted in the preparation and editing of this manuscript.

Charles Hall MD/PhD provided expertise on seizure induction experiments and translational impact. Moreover, Dr. Hall prepared all other sections of this manuscript.

cellular responses to seizures in the adult zebrafish brain are similar to those observed in mammalian brains.

Keywords

Epilepsy; seizures; zebrafish; neurogenesis

Introduction

One percent of the general population will have a seizure over the course of their lifetime (Hauser et al., 1991). Half of these people will go on to have recurrent and unprovoked seizures and be diagnosed with epilepsy. Nearly half of people with epilepsy will continue to have seizures despite optimum medical treatment (Shorvan, 2007; Cascino, 2008). Recurrent seizures are associated with increased rates of mortality (Zielinski, 1974), cognitive impairment (Aldenkamp, 2006), mental illness and psychosocial dysfunction (Pershad and Siddiqui, 1992) at a cost to the US economy of more than \$12 billion annually (Begley et al., 2000). Novel and more efficacious treatment paradigms for epilepsy are in desperate need.

The development of new treatment paradigms for epilepsy requires understanding how brain tissues respond to recurrent seizure activity. Much of what is known about the cellular consequences of seizure has come from rodent models, in which seizures are pharmacologically or electrically induced. Cellular responses to recurrent seizures in rodents include inflammation (Minami et al., 1991; Plata-Salamán et al., 2000), neural proliferation (Parent, 2002; Parent and Lowenstein, 2002; Zhao and Overstreet-Wadiche 2008; Vezzani, A., Friedman and Dingledine 2013) and gliosis (Glo, 1973; Rakhade and Jensen, 2009; Khurgel and Ivy, 1996). Each of these processes is a potential therapeutic target for prevention of seizures in epilepsy, but little is known about which process or combination of processes would be optimum.

Zebrafish have recently emerged as a potentially powerful model to study seizures. Like rodents, zebrafish have completely tractable genetics, vertebrate neural architecture, and are used to study many human disorders, including seizures (Baraban et al., 2005; Pineda et al., 2011; Stewart et al., 2012). Advantages of the zebrafish model over rodents include more cost effective generation and maintenance of animal lines, potentially allowing for greater amenability to high throughput, stringently controlled experimentation. The goal of this study is to determine how the adult zebrafish brain responds to recurrent seizures at the cellular level, and whether these responses are similar to those observed in mammalian models. To that end, we subjected zebrafish to pentylenetetrazole (PTZ)-induced seizures and looked for histological evidence of local inflammation with vasodilation, neural proliferation and reactive gliosis in response to recurrent seizures. No known model for inducing spontaneous seizures in zebrafish exists. However, if the zebrafish brain shows cellular responses to seizures that have been observed in mammals, then the adult zebrafish model may be applicable for studying seizure and potentially epilepsy, which in turn, could accelerate research in this crucial area.

Experimental Procedures

Animal care and use

All experiments made use of adult laboratory strain WIK zebrafish (9–14 months old). All protocols and procedures were approved by The Ohio State University's Institutional Animal Care and Use Committee. Fish were maintained in a recirculating water system (Pentair) and fed to satiety twice daily on weekdays, once daily on weekends. All experiments were performed 2–6 hours after a weekday morning feeding. *Tg (Tg(Fli:GFP))* were kindly provided by Dr. Brant Weinstein (Lawson and Weinstein, 2002) and *Tg(GFAP:GFP)* were kindly provided by Dr. Pamela Raymond (Bernados and Raymond, 2006). In total, 85 zebrafish were used in the study, in which between 3–5 fish were used per seizure or no seizure condition.

Seizure induction

As described in Pineda et al. 2010, seizures were induced by immersing free swimming zebrafish into a finger bowl containing 15 mM PTZ (Sigma Aldrich) until stage III behavioral seizure activity was observed (loss of posture with rolling to the side and clonic convulsive movements). In all seizure induction conditions, zebrafish were subjected to five once-daily PTZ-induced seizures.

Immunohistochemistry

Adult zebrafish were anesthetized using tricaine and were fixed in paraformaldehyde overnight and brains were dissected out the following day, followed by cryoprotection overnight (30% sucrose in PBS). Brains were embedded in OCT, and cryosectioning was performed (18 thick). Cryosections were washed in 0.2% triton in PBS (PBST) for 10 minutes, then blocked in 10% normal goat serum in PBST for 30 minutes and were incubated with primary antibodies diluted in 2.5% normal goat serum, 1% BSA, 1% DMSO in PBST (PBDT) overnight at 40C. The following day, sections were washed in PBST (3×, 10 minutes). Sections were then incubated with secondary antibodies (diluted in PBDT) for 2 hours at room temperature, followed by washes with PBST (3X, 10 minutes). Slides were then mounted (using Dako fluorescent mounting medium) and coverslipped. Confocal images were acquired with a Leica confocal microscope. Images were processed using Fiji.

PCNA labeling: For PCNA labeling, the sections were pre-incubated in PBS at 99°C for 8 minutes and cooled down to room temperature over 15 minutes before blocking with 10% normal goat serum in PBST. The following primary antibodies were used: anti-GFP (chicken, ab13970, 1:2500, Abcam), anti-HuC/D (mouse, A-21271, 1:1000, Invitrogen), anti-GFAP (mouse, ZRF1, 1:1000, Zfin), anti-L plastin (rabbit, 1:200, a gift from Dr. Wen, University of Hong Kong, Hong Kong), anti-PCNA (mouse, PC10, 1:500, Dako). The following secondary antibodies were used (Alexa, Invitrogen, 1:500 dilution): anti-chicken 488, anti-mouse 568, anti-rabbit 647.

EdU injection and immunohistochemistry

To label cells in S-phase of the cell cycle, adult zebrafish were anesthetized with tricaine and injected intraperitoneally with 20 µL of 10 mM solution of 5-ethynyl-2'-deoxyuridine (EdU)

diluted in PBS. For an extended pulse of EdU, fish were injected twice a day with 4 hours between injections for 2 days, and subjected to chase periods ranging from 1 day following the last injection to 5, 14, 21 and 90 days. EdU staining was conducted using The Click-iT® EdU Alexa Fluor® 555 Imaging Kit (Invitrogen) according to the manufacturer's protocol. The sections were washed twice with PBS and then incubated with a Click-iT™ reaction cocktail containing Click-iT™ reaction buffer, CuSO₄, Alexa Fluor® 555 Azide, and reaction buffer additive for 5 minutes while protected from light. The sections were washed once more with PBS. For subsequent antibody staining, sections were washed once with PBST and then incubated with 10% normal goat serum in PBST then followed the staining step above. The slides were then washed twice with PBST and coverslipped with Vectashield mounting media (Vector Laboratories Inc, CA). All steps were carried out at room temperature.

Data quantification and statistical analysis

Tissue sections (18 µm) between the olfactory bulb and the caudal telencephalon were used for statistical analyses. For quantification of cell number, reactive gliosis, and blood vessel diameter, cryosections of similar dorsal telencephalon regions were analyzed. Both sides of the telencephalon (left and right side) were analyzed. For cell counts, average value is displayed as number of cells per section, which includes the sum of cells from both sides. To quantify reactive gliosis, FIJI was used to measure density of GFAP+ staining in similar dorsal telencephalon regions with the same area dimensions (settings: x=0, y=0, w=512, h=290). To analyze vasculature dilation, FIJI was used to measure the diameters of blood vessels in the dorsal telencephalon.

Co-localization was verified by analyzing high-resolution confocal stacks. The optical sections were taken at 1 µm intervals using a 40X objective. Microsoft Excel was used to process the measured data. Graphpad Prism was used to generate graphs and analyze statistics. We opted to use non-parametric statistical tests since it is difficult to test for normal distribution without large sample sizes. To analyze significance among more than two groups, *P* values were determined using the Kruskal-Wallis test followed by Dunn's test to correct for multiple comparisons. To analyze significance among two groups, *P* values were determined using the Mann-Whitney test. ****P* 0.001; ***P* 0.01; **P* 0.05. *P*>0.05 was not considered significant (ns). Statistics described in the results are mean ±SEM.

All measurements were made by an experimenter blind to the experimental condition.

Results

Adult zebrafish exhibit post-seizure vasodilation and leukocytosis

To analyze the effect of seizure activity on the adult zebrafish brain, we exposed animals to PTZ, a proconvulsant that induces seizure activity by inhibition of the gamma-amino-butyric-acid receptor type A (GABA-A) (Huang et al., 2001). Adult zebrafish were subjected to five consecutive once-daily seizures then analyzed 1, 5, 14 and 21 days after the fifth seizure. This paradigm was used throughout this study.

We first looked for changes in the cerebral vasculature and inflammation, as increased inflammation and vasculature changes have been documented in rodent seizure models (Minami et al., 1991; Plata-Salaman et al., 2000; Rigau et al., 2007; Fabene et al., 2008). Inflammation has also been observed in adult zebrafish brain injury conditions (Kroehne et al., 2011; Kyritsis et al., 2012). To characterize vasculature changes, we examined the diameter of blood vessels in the zebrafish brain (Fig. 1) after exposure to PTZ as described and compared these diameters to those of seizure naïve fish. To identify blood vessels, we obtained a line of zebrafish expressing green fluorescent protein (GFP) under control of the zebrafish friend leukemia integration 1 transcription factor (FLI1) promoter (*Tg(FLI1:GFP)*) (Lawson and Weinstein, 2002). *FLI1* is an endothelial specific promoter which when coupled to GFP, allows for immediate visualization of vasculature using fluorescent microscopy (Fig. 2 A–D). We focused on blood vessels in and around the lateral part of the dorsal telencephalon (DI), the teleost equivalent to the hippocampus (Fig. 1; Mueller et al., 2012; Portavella et al., 2004; Rupp 1996). We observed a significant increase in blood vessel diameter at post-seizure day 1 ($6.97 \pm 0.35 \mu\text{m}$, $n=5$) compared to the no seizure group ($3.70 \pm 0.22 \mu\text{m}$, $n=3$, $P < 0.05$) (Fig. 2 B, E). By post seizure day 14, blood vessel diameter ($3.62 \pm 0.05 \mu\text{m}$, $n=3$) was comparable to the no seizure group ($3.70 \pm 0.22 \mu\text{m}$, $n=3$, $P > 0.05$) (Fig. 2 C–E). To characterize inflammation, we stained with L-plastin antibody, a cytosolic protein specific to lymphocytes (Herbomel et al., 1999; Jin et al., 2009). We found increased number of leukocytes in the brain parenchyma at day 1 post seizure (43 ± 4 cells, $n=5$, $P < 0.05$) that normalized to no seizure levels (6 ± 1 cells, $n=3$) by day 14 post seizure (9 ± 3 cells, $n=3$, $P > 0.05$) (Fig. 2 A–D, F). Collectively, these results show that recurrent seizures alter the vasculature structure and increase inflammation, which are similar to the vasculature alterations and inflammatory responses observed in mammalian seizures (Minami et al., 1991; Plata-Salaman et al., 2000; Rigau et al., 2007; Fabene et al., 2008) as well as the upregulated inflammatory response in zebrafish injury (Kroehne et al., 2011; Kyritsis et al., 2012).

Leukocyte proliferate in response to recurrent seizures

As described above, we found increased leukocytes in the brain parenchyma in response to seizures. To determine whether the increased numbers of leukocytes was due to proliferation, we subjected adult *Tg(FLI1:GFP)* zebrafish to recurrent PTZ-seizures and looked for proliferating leukocytes in the cerebral vasculature one day post seizure. We then processed sections from the brains of these animals and used an antibody against proliferating cell nuclear antigen (PCNA), a processivity factor for eukaryotic DNA polymerase that is abundant in mitotic cells (Kubben et al., 1994), to identify proliferating cells. At day 1 post seizure, of all L-plastin+ cells, $94 \pm 1\%$ ($n=5$) also co-localized with PCNA (Fig. 3 B, C). We also found that $87 \pm 1\%$ ($n=5$) of L-plastin+ cells closely associated with Fli+ blood vessels (Fig. 3 B,C). $70 \pm 2\%$ ($n=5$) of L-plastin+ cells are both PCNA+ and closely associated with Fli+ blood vessels (Fig. 3B,C). In contrast, we found none or little co-localization between L-plastin and PCNA or close association between L-plastin+ cells and Fli+ blood vessels (Fig. 3 A). These results indicate the presence of intravascular leukocyte proliferation that seem to lead to increased total number of leukocytes immediately following seizures.

Seizure-induced cellular proliferation in the teleost equivalent of the hippocampus

Increased cell proliferation has been observed following drug-induced seizures in rodents and brain injury in adult zebrafish (Parent et al., 1997; Stringer 1996; Kroehne et al., 2011; Skaggs et al., 2015). To determine whether drug-induced seizures increase cellular proliferation in adult zebrafish, we subjected populations of zebrafish to consecutive PTZ-induced seizures and analyzed cellular proliferation using PCNA. We counted cells in the ventricular zone and the parenchyma of the DI and found significantly increased number of PCNA+ cells one day after seizures (85 ± 9 cells, $n=3$, ventricular zone; 43 ± 10 cells, $n=3$, parenchyma) with respect to no seizure fish in both the ventricular zone (22 ± 3 cells, $n=5$, $P < 0.05$) and parenchyma (7 ± 1 cells, $n=5$, $P < 0.05$) of the DI (Fig. 4 A, B, D, E). The effect returned to baseline by day 21 after seizures (24 ± 2 cells, $n=3$, ventricular zone; 9 ± 2 cells, $n=3$, parenchyma; $P > 0.05$) (Fig. 4 C, D, E). These results suggest that recurrent seizures lead to increased cellular proliferation in the ventricular zone and the parenchyma of the DI, mimicking the upregulated proliferation seen in rodents following drug-induced seizures and in zebrafish following brain injury (Parent et al., 1997; Stringer 1996; Kroehne et al., 2011; Skaggs et al., 2015).

Seizure-induced neurogenesis in the zebrafish ventricular zone of the DI region

Drug-induced seizures in rodents lead to increased adult neurogenesis in the hippocampus (Parent et al., 1997; Liu et al., 2008). Increased neurogenesis has also been observed in the zebrafish in response to injury (Kroehne et al., 2011; Skaggs et al., 2015). To determine whether recurrent seizures lead to more neurogenesis in zebrafish, we performed a pulse chase study using 5-ethynyl-2'-deoxyuridine (EdU) to identify newly synthesized DNA and analyzed for co-localization with the pan-neuronal specific markers HuC/D on post seizure days 1, 5, 14, 21 and 90 (Fig. 5). One day after seizures, the number of EdU+/HuC/D+ cells was not different between the no seizure group (2.3 ± 0.3 cells, $n=3$) and the seizure group (2.7 ± 0.9 cells, $n=3$, $P < 0.05$). EdU+/HuC/D+ cells were significantly higher in fish that had experienced seizures with respect to on days 5 (17 ± 4 cells, $n=4$ vs 152 ± 3 cells, $n=5$, $P < 0.05$), 14 (11 ± 3 cells, $n=5$ vs 168 ± 9 cells, $n=4$, $P < 0.05$), and 21 (13 ± 1 cells, $n=4$ vs 175 ± 2 cells, $n=5$, $P < 0.05$). There seemed to be a trend towards more EdU+/HuC/D+ at 90 days after seizures (9 ± 2 cells, $n=3$) compared to no seizure group (5 ± 1 cells, $n=3$), but the effect was not statistically significant ($P > 0.05$) (Fig. 5 F). Moreover, double positive cells were present within the ventricular zone of the DI, but not in the parenchyma (Fig. 5 D arrows, E arrowheads). These results indicate that recurrent drug-induced seizures increase adult neurogenesis in the ventricular zone of the zebrafish hippocampal-like region.

Radial-glia like cells - a potential source of neuro-progenitors

In the adult mammalian and zebrafish brain, glial fibrillary acidic protein (GFAP) positive radial glia-like cells are the primary neuronal progenitors that develop into adult-born neurons (Doetsch et al., 1999; Seri et al., 2001; Kroehne et al., 2011). To identify radial-glia like cells, we obtained a transgenic fish in which GFP is driven by zebrafish *gfap* promoter elements, which allow for direct visualization of glial cells under fluorescent microscopy (Bernados and Raymond, 2006). As described above, we subjected a population of *Tg(gfap:GFP)* fish to recurrent seizures and injected the fish with EdU to label actively

dividing cells and detected neurons by anti HuC/D labeling (Fig. 6). We found localization of EdU with GFAP in the ventricular zone suggesting the proliferation of radial glia-like cells. These cells did not express HuC/D indicating that at this juncture, they are in fact glial cells and not differentiated neurons. The presence of proliferating glia in the ventricular zone suggests that the neural proliferation described above originates from radial glia-like cells in the zebrafish, much in the same way that drug-induced seizures stimulate proliferation of GFAP+ radial glia-like in rodents (Parent et al., 1997; Hüttman et al., 2003). Moreover, new neurons observed following brain injury in fish are known to be derived from radial glia-like cells (Kroehne et al., 2011). Given the similarity of inflammatory and proliferative responses between our drug-induced seizure studies and brain injury in zebrafish thus far, it is likely that the newborn neurons observed following seizures here may have also been derived from radial glia-like cells.

Reactive gliosis after PTZ-seizures

In the mammalian brain, reactive gliosis is characterized by increased GFAP expression and hypertrophy of glial processes observed in rodents following drug-induced seizures and human epilepsy, as well as in adult zebrafish following traumatic injury (Stringer et al., 1996; Sommer 1880; Nascimento et al., 2012; Kroehne et al., 2011). To determine whether zebrafish experience diffuse reactive gliosis in response to seizures, we subjected adult zebrafish to recurrent seizures and compared GFAP antibody staining to seizure-naïve fish (Fig. 7). We found increased GFAP labeling by post-seizure day 5 ($2717 \pm 163 \times 10^4$ pixels, $n=4$) compared to the no seizure group ($391 \pm 5 \times 10^4$ pixels, $n=3$, $P < 0.05$) (Fig. 7 A, B). This effect decreased to no seizure levels by post seizure day 14 ($348 \pm 50 \times 10^4$ pixels, $n=4$, $P > 0.05$) (Fig. 7 C, D, E). These results suggest that reactive gliosis, a commonly observed histological manifestation of recurrent seizures in rodents and brain injury in zebrafish, also occurs after PTZ-induced seizures in adult zebrafish.

Discussion

Basic research into epilepsy has mainly depended on mammalian rodent animal models. Epilepsy is experimentally modeled in rodents by pharmacological induction of seizures using convulsant agents. This rodent epilepsy model has been used extensively over the past several decades to study the disease mechanisms of epilepsy as well as preclinical development of anti-epileptic drugs (AEDs), the mainstay epilepsy treatment. However, AEDs are still ineffective in up to 50% of patients and induce serious drug-related side effects (Shorvan, 2007; Cascino, 2008). The lack of a truly satisfactory treatment for epilepsy compels the need to explore alternative epilepsy and seizure models.

The zebrafish has recently emerged as a potentially powerful model to study epilepsy. It has previously been shown that PTZ induces tonic-clonic-like seizures as well as ictal and interictal-like electrical activity in larval zebrafish that would be expected from a rodent seizure model (Baraban, 2005; Pineda et al., 2011; Meyer et al., 2016). PTZ has also been utilized successfully to induce tonic-clonic-like seizures in the adult zebrafish (Mussulini et al., 2013). These works suggest that the zebrafish model recapitulates the behavioral

manifestation of seizures and abnormal electrical brain activity observed in mammalian seizures, and therefore can be a powerful alternative model for seizure research.

Despite several lines of evidence showing that the zebrafish model recapitulates the behavioral and electrical characteristics of mammalian seizures, there is no published study, to the best of our knowledge, that characterizes the histological features of any larval or adult zebrafish seizure model. We therefore sought to characterize the cellular responses to recurrent PTZ-induced seizures in the adult zebrafish model to determine whether the zebrafish model also recapitulates the cellular remodeling following seizures observed in mammalian epilepsy. Our study shows that in response to recurrent PTZ-induced seizures, the zebrafish brain exhibited neurovascular alterations, inflammation, increased neurogenesis, and reactive gliosis. All of these described cellular changes have been documented in rodent seizure models. Altogether, our results provide evidence that the zebrafish model recapitulates histopathological features observed in mammalian seizure models.

To what extent do the cellular responses observed in PTZ-induced seizures represent epileptogenesis? Strictly speaking, epileptogenesis renders the brain capable of generating spontaneous seizures. To date, there is no published observation of spontaneous seizures in zebrafish, including our study here. Every seizure episode had to be drug-induced, in contrary to rodent models, in which a single administration of a pro-convulsant drug results in status epilepticus that then leads to spontaneous seizures. Therefore, the upregulated neurogenesis and inflammation observed in our study may simply represent responses to seizure activity rather than a part of epileptogenesis, especially given that these cellular responses normalize to background levels after PTZ-induced seizures. While cell proliferation does normalize to background levels in rodent models with spontaneous seizures (Parent et al., 1997), reactive gliosis appears to be chronically activated and is often observed in human medial temporal lobe epilepsy (Nascimento et al., 2012; Sommer 1880). On the other hand, the reactive gliosis in response to PTZ-induced seizures in zebrafish normalized to background levels within two weeks. Therefore, our interpretation is that the increased neurogenesis, inflammation, and reactive gliosis observed here are at least the brain's response to seizure activity, but can potentially result in epileptogenesis if the cellular responses persist chronically as in some cases observed in mammalian seizure models.

Why might the cellular responses to seizures in the zebrafish brain normalize quickly, whereas some of those in rodent brains seem to manifest chronically? It is known that zebrafish brains have higher regenerative capacity than mammalian brains. In response to traumatic brain injury, the adult zebrafish brain is able to restore normal morphology, generate newborn neurons that integrate into neural circuitries, and avoid the formation of a glial scar (Kroehne et al., 2011; Skaggs et al., 2015; Schmidt et al., 2013). Traumatic brain injury in rodent brain also increases cell proliferation, but the traumatic insult leaves a glial scar and no newborn neurons have been found in response to injury (Buffo et al. 2008; Buffo et al., 2005). A higher regenerative capacity in zebrafish brain might therefore allow it to recover from the cellular consequences of seizures better than mammalian brain. As a result, zebrafish may never become a model of spontaneous seizure since the zebrafish brain has

better neuroprotective mechanisms. If such is the case, understanding what makes the zebrafish brain recover better from seizures may reveal novel therapeutic avenues to prevent epileptogenesis in humans.

On the other hand, it is worth noting that in this study the PTZ-induced tonic-clonic-like seizures last for several seconds, whereas drug-induced seizures in rodents are status epilepticus in which seizures last for several hours. Therefore, it may also be possible that the endogenous regenerative response to seizures is similar between mammals and zebrafish, but the regenerative response is not robust enough to recover from the more severe seizures in rodents. It would be crucial for future studies to induce status epilepticus in adult zebrafish and determine whether the fish brain can still recover from more severe seizures or develop spontaneous seizures similar to those in rodent models. Part of the reason that accounts for the lack of status epilepticus studies in zebrafish is that it is difficult to keep a sufficient number of animals alive following long duration of seizures for experimental studies. Indeed, rodents subjected to drug-induced status epilepticus often die due to the harsh seizure treatment.

Taken together, our study shows that the adult zebrafish brain responds to drug-induced seizures in the same manner as mammalian brains. This study lays the foundation for future work to further characterize the similarities and/or differences between the zebrafish and mammalian seizure models. If future studies show that more severe seizure types, such as status epilepticus, also induce spontaneous seizures in zebrafish, then zebrafish might become a suitable model for not only understanding the cellular consequences to seizure activity but also epileptogenesis. On the other hand, if future work shows that zebrafish fail to develop spontaneous seizures after epilepticus, then a greater insight into the enhanced neural regeneration of the adult zebrafish brain may provide clues to develop therapeutic methods that prevent epileptogenesis.

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References

- Aldenkamp AP. Cognitive impairment in epilepsy: State of affairs and clinical relevance. *Seizure*. 2006; 15:219–220.
- Baraban SC, Taylor MR, Castro PA, Baier H. Pentylentetrazole induced changes in zebrafish behavior, neural activity and c-fos expression. *Neuroscience*. 2005; 131:759–768. [PubMed: 15730879]
- Begley CE, Famulari M, Annegers JF, Lairson DR, Reynolds TF, Coan S, Rocca WA. The Cost of Epilepsy in the United States: An Estimate from Population-Based Clinical and Survey Data. *Epilepsia*. 2000; 41(3):342–351. [PubMed: 10714408]
- Buffo A, Vosko MR, Ertürk D, Hamann GF, Jucker M, Rowitch D, Götz M. Expression pattern of the transcription factor Olig2 in response to brain injuries: implications for neuronal repair. *P Natl Acad Sci USA*. 2005; 102(50):18183–18188.
- Buffo A, Rite I, Tripathi P, Lepier A, Colak D, Horn AP, Götz M. Origin and progeny of reactive gliosis: A source of multipotent cells in the injured brain. *P Natl Acad Sci USA*. 2008; 105(9): 3581–3586.

- Cascino GD. When drugs and surgery don't work. *Epilepsia*. 2008; 49:79–84. [PubMed: 19087121]
- Doetsch F, Caille I, Lim DA, Garcia-Verdugo JM, Alvarez-Buylla A. Subventricular zone astrocytes are neural stem cells in the adult mammalian brain. *Cell*. 1999; 97(6):703–716. [PubMed: 10380923]
- Eid T, Brines ML, Cerami A, Spencer DD, Kim JH, Schweitzer JS, de Lanerolle NC. Increased expression of erythropoietin receptor on blood vessels in the human epileptogenic hippocampus with sclerosis. *J Neuropath Exp Neuro*. 2004; 63(1):73–83.
- Fabene PF, Mora GN, Martinello M, Rossi B, Merigo F, Ottoboni L, Constantin G. A role for leukocyte-endothelial adhesion mechanisms in epilepsy. *Nat Med*. 2008; 14:1377–1383. [PubMed: 19029985]
- Glo FL. Membrane properties of neuroglia in epileptogenic gliosis. *Brain res*. 1973; 55:159–171. [PubMed: 4713186]
- Hauser WA, Annegers JF, Kerland LT. Prevalence of epilepsy in Rochester, Minnesota. *Epilepsia*. 1991; 32:429–445. [PubMed: 1868801]
- Herbomel P, Thisse B, Thisse C. Ontogeny and behaviour of early macrophages in the zebrafish embryo. *Development*. 1999; 126(17):3735–3745. [PubMed: 10433904]
- Huang RQ, Bell-Horner CL, Dibas MI, Covey DF, Drewe JA, Dillon GH. Pentylentetrazole-induced inhibition of recombinant γ -aminobutyric acid type A (GABAA) receptors: mechanism and site of action. *J Pharm Exp Ther*. 2001; 298(3):986–995.
- Hüttmann K, Sadgrove M, Wallraff A, Hinterkeuser S, Kirchhoff F, Steinhäuser C, Gray WP. Seizures preferentially stimulate proliferation of radial glia-like astrocytes in the adult dentate gyrus: functional and immunocytochemical analysis. *Eur J Neurosci*. 2003; 18(10):2769–2778. [PubMed: 14656326]
- Jin H, Sood R, Xu J, Zhen F, English MA, Liu PP, Wen Z. Definitive hematopoietic stem/progenitor cells manifest distinct differentiation output in the zebrafish VDA and PBI. *Development*. 2009; 136(4):647–654. [PubMed: 19168679]
- Khurgel M, Ivy GO. Astrocytes in kindling: relevance to epileptogenesis. *Epilepsy res*. 1996; 26:163–175. [PubMed: 8985698]
- Kroehne V, Freudenreich D, Hans S, Kaslin J, Brand M. Regeneration of the adult zebrafish brain from neurogenic radial glia-type progenitors. *Development*. 2011; 138(22):4831–4841. [PubMed: 22007133]
- Kubben FJ, Peeters-Haesevoets A, Engels LG, Baeten CG, Schutte B, Arends JW, Blijham GH. Proliferating cell nuclear antigen (PCNA): a new marker to study human colonic cell proliferation. *Gut*. 1994; 35(4):530–535. [PubMed: 7909785]
- Lawson ND, Weinstein BM. In Vivo Imaging of Embryonic Vascular Development Using Transgenic Zebrafish. *Dev Biol*. 2002; 248(2):307–318. [PubMed: 12167406]
- Liu YWJ, Curtis MA, Gibbons HM, Mee EW, Bergin PS, Teoh HH, Faull RLM. Doublecortin expression in the normal and epileptic adult human brain. *Eur J Neurosci*. 2008; 28(11):2254–2265. [PubMed: 19046368]
- Meyer M, Dhamne SC, LaCoursiere CM, Tambunan D, Poduri A, Rotenberg A. Microarray Noninvasive Neuronal Seizure Recordings from Intact Larval Zebrafish. *PLOS ONE*. 2016; 11(6):e0156498. [PubMed: 27281339]
- Minami M, Kuraishi Y, Satoh M. Effects of kainic acid on messenger RNA levels of IL-1 β , IL-6, TNF α and LIF in the rat brain. *Biochem Biophys Res Commun*. 1991; 176(2):593–598. [PubMed: 1709015]
- Mueller T, Dong Z, Berberoglu MA, Guo S. The dorsal pallium in zebrafish, *Danio rerio* (Cyprinidae, Teleostei). *Brain res*. 2011; 1381:95–105. [PubMed: 21219890]
- Mussulini BHM, Leite CE, Zenki KC, Moro L, Baggio S, Rico EP, Campos MM. Seizures induced by pentylentetrazole in the adult zebrafish: a detailed behavioral characterization. *PLOS ONE*. 2013; 8(1):e54515. [PubMed: 23349914]
- do Nascimento AL, dos Santos NF, Pelagio FC, Teixeira SA, de Moraes Ferrari EA, Langone F. Neuronal degeneration and gliosis time-course in the mouse hippocampal formation after pilocarpine-induced status epilepticus. *Brain res*. 2012; 1470:98–110. [PubMed: 22781142]

- Parent JM, Timothy WY, Leibowitz RT, Geschwind DH, Sloviter RS, Lowenstein DH. Dentate granule cell neurogenesis is increased by seizures and contributes to aberrant network reorganization in the adult rat hippocampus. *J Neurosci.* 1997; 17(10):3727–3738. [PubMed: 9133393]
- Parent JM. The role of seizure-induced neurogenesis in epileptogenesis and brain repair. *Epilepsy res.* 2002; 50:179–189. [PubMed: 12151127]
- Parent JM, Lowenstein DH. Seizure-induced neurogenesis: are more new neurons good for an adult brain? *Progress in brain research.* 2002; 135:121–131. [PubMed: 12143334]
- Pershad D, Siddiqui RS. Psychosocial dysfunction in adults with epilepsy. *Int J Rehabil Res.* 1992; 15:258–261. [PubMed: 1428394]
- Pineda R, Beattie CE, Hall CW. Recording the adult zebrafish cerebral field potential during pentylenetetrazol seizures. *J Neurosci Methods.* 2011; 200:20–28. [PubMed: 21689682]
- Plata-Salamán CR, Ilyin SE, Turrin NP, Gayle D, Flynn MC, Romanovitch AE, McIntyre DC. Kindling modulates the IL-1 β system, TNF- α , TGF- β 1, and neuropeptide mRNAs in specific brain regions. *Brain Res Mol Brain Res.* 2000; 75(2):248–258. [PubMed: 10686345]
- Portavella M, Torres B, Salas C, Papini MR. Lesions of the medial pallium, but not of the lateral pallium, disrupt spaced-trial avoidance learning in goldfish (*Carassius auratus*) *Neurosci. let.* 2004; 362(2):75–78.
- Rakhade SN, Jensen FE. Epileptogenesis in the immature brain: emerging mechanisms. *Nat Rev Neurol.* 2009; 5:380–391. [PubMed: 19578345]
- Rigau V, Morin M, Rousset MC, de Bock F, Lebrun A, Coubes Lerner-Natoli M. Angiogenesis is associated with blood-brain barrier permeability in temporal lobe epilepsy. *Brain.* 2007; 130(7):1942–1956. [PubMed: 17533168]
- Rupp B, Reichert H, Wullmann MF. The zebrafish brain: a neuroanatomical comparison with the goldfish. *Anat Embryol.* 1996; 194:187–203. [PubMed: 8827327]
- Seri B, Garcia-Verdugo JM, McEwen BS, Alvarez-Buylla A. Astrocytes give rise to new neurons in the adult mammalian hippocampus. *J Neurosci.* 2001; 21(18):7153–7160. [PubMed: 11549726]
- Schmidt R, Strähle U, Scholpp S. Neurogenesis in zebrafish—from embryo to adult. *Neural Dev.* 2013; 8(1):1.
- Shorvon SD. The epidemiology and treatment of chronic and refractory epilepsy. *Epilepsia.* 2007; 37:S1–S3.
- Sommer W. Erkrankung des Ammonshorns als aetiologisches Moment der Epilepsie. *Eur Arch Psychiatry Clin Neurosci.* 1880; 10(3):631–675.
- Stewart AM, Desmond D, Kyzar E, Gaikwad S, Roth A, Riehl R, Collins C, Monnig L, Green J, Kalueff AV. Perspectives of zebrafish models of epilepsy: What, how and where next? *Brain Res Bull.* 2012; 87:135–143. [PubMed: 22155548]
- Stringer JL. Repeated seizures increase GFAP and vimentin in the hippocampus. *Brain res.* 1996; 717(1):147–153. [PubMed: 8738264]
- Vezzani A, Friedman A, Dingledine RJ. The role of inflammation in epileptogenesis. *Neuropharmacol.* 2013; 69:16–24.
- Zhao CS, Overstreet-Wadiche L. Integration of adult generated neurons during epileptogenesis. *Epilepsia.* 2008; 49:3–12.
- Zielinski JJ. Epilepsy and mortality rate and cause of death. *Epilepsia.* 1974; 15:191–201. [PubMed: 4525178]

Highlights

We characterized the cellular responses of the adult zebrafish brain to seizures

Recurrent drug-induced seizures led to acute CNS inflammation characterized by vascular and leukocytic alterations

We also found an upregulation of adult neurogenesis and reactive gliosis following seizures

Seizures in zebrafish recapitulate histological features of mammalian seizures

Figure 1.

Schematic of zebrafish brain shown in sagittal view (top) and in axial cross section (bottom row). OB – olfactory bulb; TEL – telencephalon; IL – intermediolateral area; CER – cerebellum; MED – medulla; Dm – dorsomedial pallium; Dc – dorsocentral pallium; Dl – lateral part of the dorsal telencephalon; VZ – ventricular zone.

Figure 2.

Blood vessel dilation and leukocytosis in the DL of zebrafish subjected to recurrent PTZ seizures. (A–D) Coronal sections of the DL in adult transgenic zebrafish with green fluorescent protein driven by endothelial specific friend leukemia antigen (Tg(fli:GFP)) and antibody labeling of leukocyte specific L-plastin. Zoomed-in pictures are also shown (white box shows zoomed-in area). A) No seizure fish (No S) showing no leukocyte co-localization with blood vessels. B) Sections from a fish 1 day after seizures (1d S) showing increased co-localization of leukocyte antibody staining with endothelial GFP. C) 5 days after seizures (5d

S) showing the response beginning to return to baseline. D) 14 days after seizures (14d S) showing maintenance of baseline. E) Measurement of the blood vessel diameter revealed acute dilation of vessels at 1 day following seizures (1d S). Blood vessel diameter returned to baseline levels by 14 days after seizures (14 d S). F) L-plastin+ leukocytes were acutely upregulated at 1 day after seizures and returned to baseline levels at 14 days post seizures. * $P < 0.05$. Statistical significance determined by Kruskal-Wallis test followed by Dunn's test. Scale bar 75 μm . Scale bar 25 μm in zoomed in pictures. Median and individual data points are shown for each group.

Figure 3. Proliferating L-plastin+ leukocytes closely associated with cerebral blood vessels 1 day following seizures (1d S). (A–B) Coronal sections of the DI in adult Tg(fli:GFP) transgenic zebrafish driving expression of GFP in blood vessels stained with proliferative marker PCNA and leukocyte marker L-plastin. A) No seizure control. Few L-plastin+ cells were found, and none of the present L-plastin+ cells colocalized with PCNA. B) 1 day after seizures (1d S), PCNA+ cells in the parenchyma of the DI were found adjacent to blood vessels (yellow arrow). A subset of these blood vessel-associated PCNA+ cells colocalized with L-plastin (white arrow), indicating the presence of proliferating leukocytes near cerebral blood vessels. Scale bar 25 μ m. Median and individual data points are shown for each group.

Figure 4.

Upregulation of cell proliferation in the adult zebrafish DL following seizures. (A–C) Coronal sections of the adult zebrafish DL stained with anti-PCNA, a proliferative marker. A) Baseline mitotic activity in the telencephalon of a no seizure control adult zebrafish (no S). B) Increased cell proliferation at 1 day post recurrent seizures (1d S) C) Reduction of proliferative activity at 21 days post recurrent seizures (21d S). D) PCNA+ cell count in the ventricular zone of the DL, a hippocampal-like region in the zebrafish, indicates that cell proliferation peaked at 1 day post recurrent seizures and returned to baseline level at 21 days

post recurrent seizures. E) Similar to ventricular zone of the DL, PCNA+ cell count in the parenchyma region also shows peak proliferative activity at 1 day post recurrent seizures (1d S). Unregulated cell proliferation returned to baseline levels at 21 days post recurrent seizures (21d S). * $P < 0.05$. Statistical significance was determined by the Kruskal-Wallis test followed by Dunn's test. Scale bar 75 μm . Median and individual data points are shown for each group.

Figure 5. Seizure-induced neurogenesis in the adult zebrafish hippocampal-like region. Adult zebrafish received a total of 4 EdU injections on the 4th and 5th seizures (2 injections per day), which were periods of high proliferative activity as suggested by the previous PCNA staining experiment. Seizure-naïve control adult zebrafish received EdU injections on two consecutive days. Animals in both groups were sacrificed 1, 5, 14, 21, and 90 days following the last EdU injection for co-labeling analysis with anti-HuC/D, a marker of differentiated neurons. Note the presence of EdU+/HuCD+ double positive in the ventricular zone of the

DI, whereas the parenchyma of the DI showed no co-localization between EdU and HuC/D. (A–B) Coronal sections of the DI in EdU-injected animals with EdU (red) and anti-HuC/D (green) immunohistochemistry killed at 1, 5, 21, and 90 days after the last EdU injection. A) No seizure control. B) Seizure group. (C–E) Zoomed-in view of the ventricular zone and parenchyma in EdU-injected animals killed at 5 days after the last EdU injection, detailing EdU+ cells co-localized with HuC/D in the ventricular zone but not in the parenchyma. C) Ventricular zone in no seizure control. D) Ventricular zone in seizure group at high magnification. E) A parenchymal section in seizure group at high magnification (boxed areas of E). F) Cell count in the ventricular zone of the DI shows that most EdU+ cells co-localized with HuC/D by 21 days after seizures, and that the number of EdU+/HuCD+ cells was upregulated in the seizure group compared to no seizure controls. The EdU+ cells with neuronal phenotype in the ventricular zone survived for at least 90 days. * $P < 0.05$. Statistical significance determined by Mann-Whitney test. Scale bar 75 μm . Scale bar 25 μm in zoomed in pictures. Median and individual data points are shown for each group.

Figure 6.

Proliferating GFAP+ radial glial-like cells may mediate seizure-induced neurogenesis. In the adult mammalian and zebrafish brain, GFAP+ radial glial-like cells act as neural stem cells that can give rise to differentiated neurons. (A–B) Coronal sections of the DL region in adult transgenic *Tg(GFAP:GFP)* zebrafish injected with EdU and stained with anti-HuC/D. A) No-seizure control 1 day after last EdU injection without presence of GFAP+/EdU+ double positive cells. B) Colocalization (arrows) of GFAP and EdU shows proliferation of GFAP+ radial glial-like cells 1 day after last EdU injection in a zebrafish subjected to seizures, indicating that radial glial-like cells were proliferating possibly in order to drive seizure-induced neurogenesis. Scale bar 25 μ m.

Figure 7.

Seizure-induced reactive gliosis. (A–D) Coronal sections of the telencephalon stained with the glial marker anti-GFAP with detailed zoomed-in images of glial processes. A) No seizure control (No S). B) 1 day after seizures (1d S). C) 5 days after seizures (5d S). Note the increased expression of GFAP as well as swelling of glial processes (hypertrophy). D) 14 days after seizures (14d S). GFAP expression was decreased and glial processes were no longer swollen. E) Measurement of the pixel area covered by GFAP shows peaked gliosis at 5 days after seizures (5d S). Gliosis returned to baseline levels at 14 days after seizures (14d

S). * $P < 0.05$. Statistical significance was determined by the Kruskal-Wallis test followed by Dunn's test. Scale bar 200 μm . Scale bar 25 μm for zoomed in pictures. Median and individual data points are shown for each group.